Annex D1

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Inhibitors ytic Activity of Urokinase Type Plasminogen Activator

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Abstract: Urokinase type plasminogen activator (uPA) activates plasminogen to plasmin and is often associated with diseases where tissue remodeling is essential (e.g. cancer, macular degeneration, atherosclerosis). We discuss some of the mechanisms of uPA action in diseases, and evidence that some of the early uPA inhibitors can modulate the progression of these diseases. Recently, a number of research groups have discovered, with the aid of structure-based design, a new generation of uPA inhibitors. These inhibitors are much more potent and selective than their predecessors. We will review this progress here, and give particular attention to the structural rationale associated with these observed increases in potency and selectivity.

INTRODUCTION

Urokinase type plasminogen activator (uPA) is one of the two physiologically important serine proteases that can hydrolyze the zymogen plasminogen to form the active enzyme plasmin. The second serine protease is tissue plasminogen activator (tPA). These names, tPA and uPA, are somewhat of a misnomer because tPA primarily activates plasminogen within the hemodynamic spaces, while uPA primarily activates plasminogen within tissues. Once activated by either uPA or tPA, plasmin degrades fibrin, and is therefore important in the fibrin-dependent processes such as blood clotting, tissue remodeling, wound healing and inflammation [1-6]. Plasmin also degrades other components of the extracellular matrix, including laminin and fibronectin. Furthermore, plasmin can activate a number of other zymogens such as matrix metalloproteases. Because it performs many functions, the inhibition of plasmin would be expected to have a number of toxic consequences, most notably with regard to hemostasis. Seminal experiments with plasminogen knockout animals have indeed demonstrated this toxicity. These animals show a propensity towards thrombosis, and are deficient in wound healing, and have diminished macrophage and lymphocyte responses to inflammatory stimuli [7-10].

Because uPA predominates in tissues, it is thought that inhibiting uPA will have minimal toxic effects on blood clotting, but should still modulate tissue-remodeling processes. This idea is reinforced by the findings that uPA is implicated in the pathogenesis of a number of diseases that require tissue remodeling such as wound healing [11-15], cancer [5,8,9,16-18], atherosclerosis [19-23], vascular restenosis [24-27], cardiac rupture[28], and macular degeneration [29]. The fact that both the tPA and uPA knockout animals have mild phenotypes relative to the plasminogen knockout animals suggests that tPA and uPA may be able to partially compensate for each other [30-32].

uPA may also act as a growth factor, independent of its proteolytic activity. uPA binds to the uPA receptor (uPAR, CD87), and this binding is thought to focus the uPA activity into areas of active tissue remodeling. The uPAR is a glycophosphatidyl inositol linked receptor, and as such has no intracellular component [6,33-35]. In spite of this, it is clear that uPA, through uPAR, is able to activate outside-in signaling pathways that induce growth in certain cells. The signal is thought to be transmitted via integrins to which the uPA:uPAR complexes bind [36-41]. More recently, these signals have been shown to proceed through growth factor receptors such as epidermal growth factor receptor [42].

The effects of uPA have been most widely studied in the progression of cancer. Indeed, increased uPA activity is found in a wide variety of human cancers, and is often an independent poor prognostic factor [43-46]. Furthermore, the progression of experimental tumors in both plasminogen or uPA knockout animals is retarded relative to the control animals [18,47-50]. Because uPA has activities unrelated to its proteolytic activity, it is not clear that the reduction in tumor progression is related to the catalytic activity of uPA.

A number of studies have been performed examining the effects of uPA inhibitors in experimental tumors. The published results have been mixed, and even when positive, the results have not been dramatic. Amiloride, a potassium-sparing diuretic, is also a uPA inhibitor [51,52]. It has been tested independently in four laboratories with mixed results. In two of these studies, the amiloride appeared to have no effect on the growth or metastatic spread of subcutaneously implanted mouse mammary [53] or rat prostate tumors [54]. In a third study, amiloride administered in the drinking water was able to diminish the number of metastatic tumors, after an intra-jugular administration of rat mammary tumor cells. In the fourth study, amiloride was again administered in the drinking water and was able to diminish the growth of DU-145 human prostate cancer cells in immunocompromised

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mice. The other pharmacological effects (diuresis) of amiloride, combined with the modest observed anti-tumor effects, make it impossible to conclude that these effects are indeed due to inhibition of uPA.

More potent and selective uPA inhibitors (B-428 and B623, compounds **17** and **16** respectively, see below) have also shown mixed results in cancer models. In one study the compounds showed a very modest growth delay at the primary site of a subcutaneous mouse mammary tumor [53]. However, the same group showed that administration of the uPA inhibitors actually increased the number of metastases formed after injecting the tumor cells into the tail vein.

Rabbani and co-workers have also examined the effects of B-428 on experimental tumors. They have shown a modest tumor growth reduction in both rat mammary [55] and prostate tumors [56] implanted subcutaneously. They were able to show an enhancement of this effect in the mammary tumors when tamoxifen, an anti-estrogen, was added to the regime [55].

One compound, WX-UK1 (compound **11**, see below), from Wilex AG, is in Phase I human trials [57]. Rodent tumor model studies have been presented at scientific meetings (92nd Annual Meeting of the American Association for Cancer Research, 2001, abstract #371), but have yet to appear in the peer-reviewed literature. This compound is not strictly a uPA inhibitor, rather it inhibits a broad spectrum of serine proteases including uPA, plasmin, thrombin and trypsin. This may be an advantage, as there are data to suggest that inhibiting multiple proteolytic paths may be more efficacious than inhibiting uPA alone [10].

A number of investigators have been able to slow the growth or spread of tumors using uPAR inhibitors or antibodies [58-61]. These data suggest that at least part of the observed phenotype in the uPA knockout animal, where tumors grew more slowly, and metastasized less frequently [18], may be due to the lack of signaling through uPAR, and thus unrelated to the catalytic activity of uPA.

These decidedly mixed results in cancer models have fostered two lines of thought. The first supposes that improved compounds will yield better results. The second is that uPA inhibition, by itself, will be insufficient to significantly effect the progression of cancer, and therefore must be combined with some other therapy (e.g. inhibition of other proteases). The resolution of this dispute regarding the efficacy of uPA inhibition will await the advance of the new generation of inhibitors into pre-clinical rodent models and ultimately into human clinical trials.

While uPA inhibitors have been studied most extensively in cancer, there is also acute interest in uPA inhibition in other diseases, particularly where the slowing of tissue remodeling may be advantageous. The uPA deficient animals show a dramatic reduction in myocardial rupture following experimental myocardial infarction [28,62]. Keloid and scar formation [10,13,63-65], restenosis following percutaneous coronary transluminal angioplasty [24,26,66-71], as well as inhibition of macular degeneration [29,72,73] are also areas of active investigation.

The ample evidence that uPA and uPAR are involved in many disease states has led many research groups to attempt to create potent and selective uPA inhibitors. While the ultimate utility of these compounds remains unknown, significant advances in inhibitor design have recently been achieved. These advances have been enabled by the use of structure-based design. Here, after a brief discussion of the historical roots of many uPA inhibitor programs, we will review recent developments in the design of reversible competitive uPA inhibitors. We will pay particular attention to the structures of these compounds when bound into the uPA active site, and discuss how these structures can help us to understand the potencies of the compounds.

THE UPA STRUCTURE

The structure of the catalytic subunit of uPA was first described Spraggon et al. [74]. Other investigators have also solved the structure of uPA, often in complex with small molecule inhibitors [75-77]. These studies provided a structural basis for understanding the similarities and differences between uPA versus other serine proteases (e.g. thrombin, factor Xa). The structures reveal that uPA has much in common with other trypsin-like serine proteases, where all have a similar protein fold. The site of catalysis, comprising the oxyanion hole plus Ser195, His57, Asp102 (the catalytic triad) are likewise similar. Another common feature of the trypsin-like serine proteases, also present in uPA, is a deep S1 pocket, which has at its base, a negative charge (the S1 pocket of the enzyme binds the amino acid, P1, on the N-terminal side of the scissile bond of the peptide substrate).

The work of Spraggon and coworkers [74] illustrated the binding of a covalent inhibitor, Glu-Gly-Arg-chloromethyl ketone (Glu-Gly-Arg-cmk), within the active site of uPA (Fig. 1). Here, the arginine side chain points towards the Asp189 carboxylate side chain at the base of the S1 subsite, presumably mimicking the natural substrate. In addition to Asp189, the guanido group of arginine also makes hydrogen bonds with the Ser190 side chain oxygen and the backbone carbonyl oxygen of Gly218. Studies described by Nienaber et al. [76] and Katz et al. [77] illustrated the binding of small molecules, such as amiloride, phenylguanidines, and amidines to uPA. The amidine or guanidine moieties are critical for the potency of these inhibitors and bind in a fashion similar to the arginine side chain (Fig. 2). Although similarities in the S1 sites predominate in the trypsin-like serine proteases, Katz and co-workers [78] have been able to identify and exploit differences at residue 190, adjacent to the Asp189 (see below). Enzymes such as uPA, trypsin and plasmin all have a Serine at position 190, whereas tPA, thrombin and factor Xa have an alanine at this position.

There are also other important differences between uPA and most trypsin-like serine proteases. In particular, the S2 and S4 subsites are smaller in uPA than in other trypsin-like serine proteases such as thrombin or factor Xa. This structural feature is due in part to a two-residue insertion at His99 that effectively reduces the size of the S2 and S4 pockets. For this reason, the peptidomimetic strategies successfully employed in the creation of thrombin inhibitors has proven more difficult in the design of uPA inhibitors.

In addition to the substrate-binding groove, there are several other subsites within the active site of uPA that are

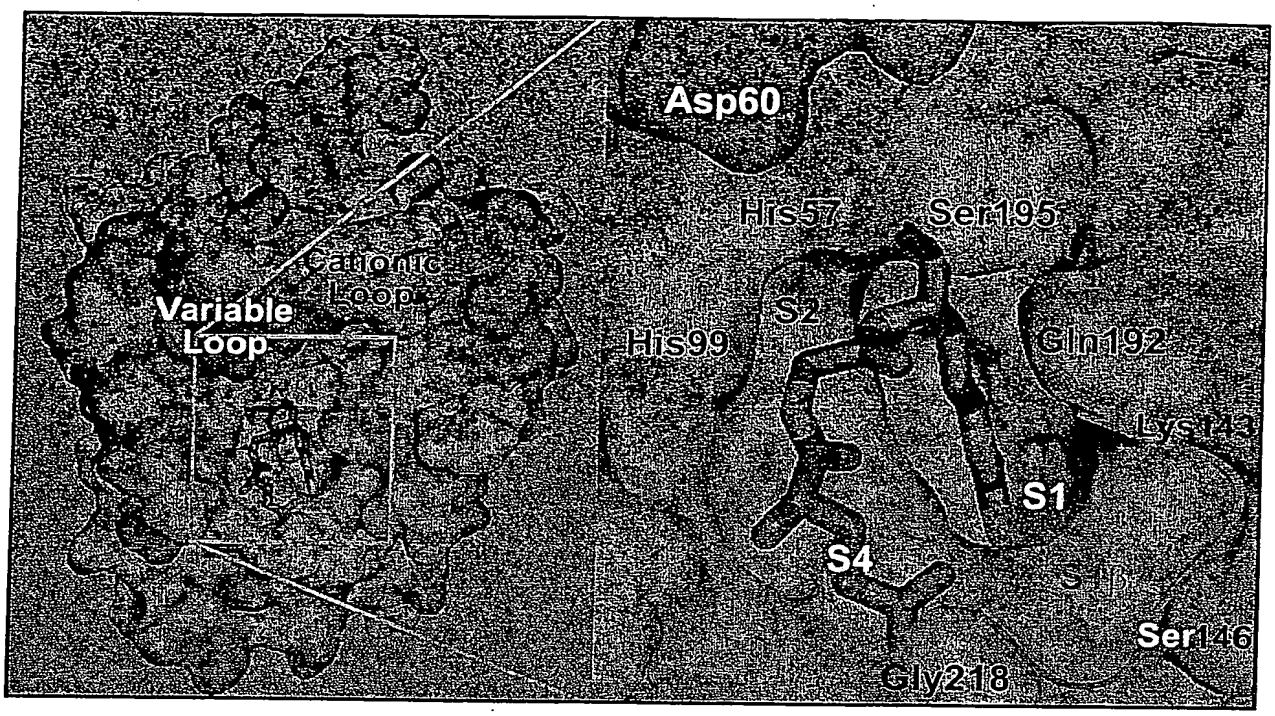


Fig. (1). The electrostatic surface of low-molecular weight uPA is depicted. Red color indicates a negative charge potential, blue positive. Glu-Gly-Arg chloromethyl ketone is docked into the active site. The structure shown here has the side chain of Gln192 rotated away from the S1β pocket. This residue is known to be rotated into different conformation depending on the bound inhibitor. The Arg residue binds into the deep S1 sub-site, and interacts with the Asp189 at the pocket's base. This critical interaction is mimicked by uPA inhibitors. Notice that the S2 and S4 subsites are relatively small, particularly when compared to other trypsin-like serine proteases.

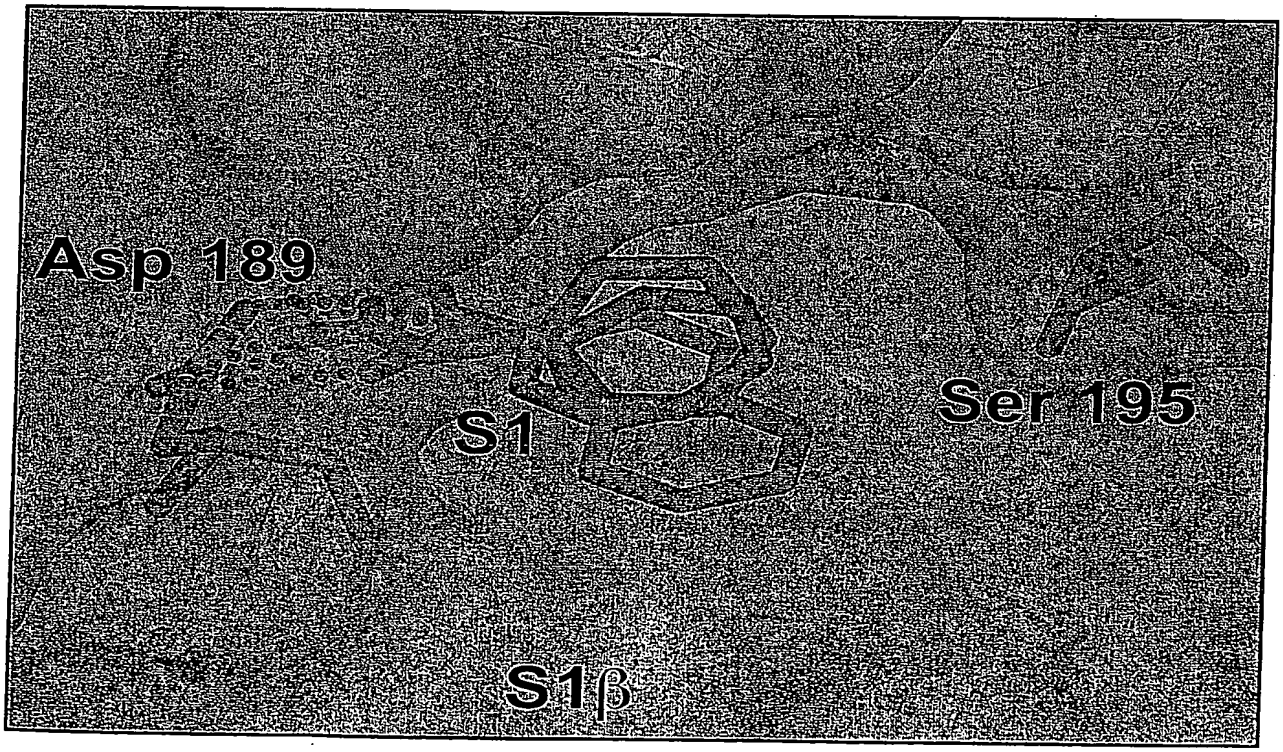


Fig. (2). Three prototype ligands are bound in the S1 subsite of uPA. Benzamidine (orange) and phenyl guanidine (magenta) both form bi-dentate hydrogen bonds with Asp189. The 2-amino group of 2-aminoisoquinoline forms a bifurcated hydrogen bond with Asp189. The advantage of the 2-aminoquinoline, as well as acetyl guanidines, is that they have a lower pKas than the amidines or guanidines and thus are likely to have better pharmacokinetic properties.

accessible to small molecule inhibitors and can be exploited to increase the potency and selectivity of uPA inhibitors. One such site is near the S1 pocket and is called the S1β site (Fig. 1). The floor of S1β is bounded by a disulfide bridge between Cys191 and Cys220. The sides of this site are defined by the residues Gly218 and Ser146, and the side chains both of Lys143 and Gln192 [76]. The S1β is not apparent in all structures because the side chain of Gln192 is mobile and can rotate to close off a large portion of the S1β site. The amino acids at both positions 143 and 146 vary considerably between serine proteases, and therefore interactions with the S1β pocket afford the potential for creating selective inhibitors.

The sequence between residues 58 and 64 is unique for a particular serine protease and has been called the variable loop. This loop is near the catalytic triad and has been exploited to design selective compounds. Not only is the variable loop significantly different between uPA and other serine proteases, it is also significantly different between the human and mouse uPAs [79]. These differences are substantial, and can be exploited to create mouse or human selective uPA inhibitors. Of course, this also means that investigators must be cautious when interpreting the results of their inhibitors when used in mixed systems (e.g. tumor xenografts models).

Active pursuit of small molecule uPA inhibitors began with the discovery that aryl guanidines, aryl amidines, or acyl guanidines all exhibited inhibition of the uPA catalytic domain. Initial compounds in each class exhibited modest potency and poor selectivity. Early chemical studies allowed the design of small molecule uPA inhibitors with improved potency and selectivity in each class. The subsequent structural studies of small molecule uPA inhibitor-enzyme complexes allowed the intelligent elaboration of these compounds to produce very potent and selective inhibitors. Structures of these potent compounds have been used to define and understand other interactions, which have been used in an iterative fashion to further improve the molecules.

ARYL GUANIDINES

Phenylguanidine (Fig. 2), inhibits uPA with a K_i of approximately 25 μM and exhibits selectivity over plasmin, thrombin, tPA, and plasma kallikrein (Table 1) [76,80,81]. Yang and coworkers [80] described a series of ortho, meta or para substituted phenylguanidine-based uPA inhibitors and proposed a model of binding for substituted phenylguanidines based on quantitative structure-activity relationship (QSAR) analysis. The most potent inhibitors were, 4-Chloro phenylguanidine (**1**, K_i = 6.1 μM) and 4-Trifluoromethyl phenylguanidine (**2**, K_i = 6.5 μM). These compounds also exhibited 10 to 20-fold selectivity for uPA over trypsin and greater than 100 to 1000-fold selectivity for uPA over plasmin, thrombin, plasma kallikrein and tPA (Table 1). Para-substituted phenyl guanidines appeared to have greater inhibitory potency when the para substituent was electron-withdrawing and hydrophobic, while bulky substituents decreased the potency of uPA inhibition. The ortho and meta substituted phenylguanidine analogs were approximately 10 to 100-fold less potent than the para-substituted compounds. Bulky substituents, especially ortho to the guanidine moiety, decreased further the uPA inhibitory potency.

The crystal structure of phenylguanidine has the expected interaction between the guanido group and the Asp189 at the base of the S1 pocket. A para position substituent on the phenylguanidine would be directed towards the catalytic triad. The para position is only 4 Angstroms from the catalytic Ser195 oxygen and is near the oxyanion hole. The improved potency of the 4-Cl phenylguanidine analog compared to phenylguanidine may be explained by a favorable interaction of the 4-Cl group with Ser195.

Despite the initial structure-activity relationships suggesting that substitution of the ortho or meta sites do not lead to improved potency, the crystal structures of these compounds reveal that this need not be true. The structures show that the aromatic moiety of the guanidine is sufficiently exposed at the ortho and para positions that substitutions at these positions may yield increased potency. Both of these positions point towards the S1β pocket, with the ortho position more favorable than the para.

Recently, another 4-substituted phenylguanidine analog (**3**) has been described by Sperl *et al.* [81,82]. The uPA inhibition for **3** is 2.4 μM. Crystallographic analysis of this compound shows the phenylguanidine moiety characteristically occupying the S1 site. The urea spacer is involved in four defined hydrogen-bonding interactions. The urea carbonyl binds to the oxyanion hole making hydrogen bonds to the backbone NH groups of Gly193 and Ser195. The urea amide hydrogens form two water-mediated hydrogen bonds to the Ser214 carbonyl and the Gln192 side chain carboxamide, respectively. This network of hydrogen bonds rigidifies the urea linkage projecting the adamantyl group toward a shallow hydrophobic pocket close to the Cys42-Cys58 disulfide bond. However, despite this extensive network of interactions, the inhibitory potency of **3** is nearly equivalent to that of the more simple 4-chloro and 4-trifluoromethyl compounds.

Amiloride (**4**) was reported to inhibit uPA with a K_i = 7 μM [51,52,76]. The selectivity of amiloride for uPA over similar trypsin-like serine proteases such as plasmin, thrombin, plasma kallikrein and tPA is high, however amiloride inhibited trypsin with nearly equal potency as uPA (Table 1).

An amiloride-uPA complex structure [76] reveals that amiloride occupies the S1 binding pocket as expected with the pyrazine ring extending further out of the S1 site than does phenylguanidine, due to the additional carbonyl group within the acyl guanidine moiety. The interaction of the guanidine moiety with the Asp189 in the S1 site is similar for both the amiloride and phenylguanidine. Other interactions between amiloride and the protein include a hydrogen bond between the inhibitor 3-amino and the oxygen of the Ser195 side chain. The 6-chloro group is directed toward the hydrophobic S1β site [76].

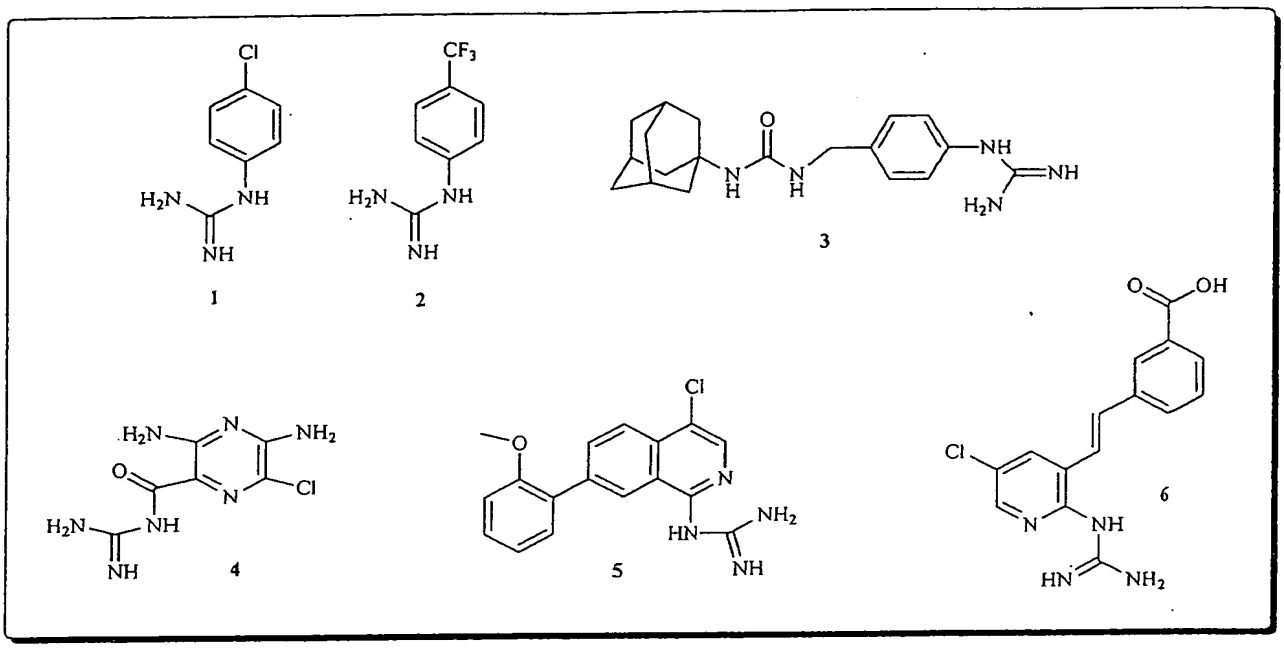
An interesting structural feature of amiloride is the acyl guanidine moiety. This moiety is less basic (pKa = 8.6) than an amidine (pKa = 10.2). For thrombin inhibitors, the high basicity of amidine or guanidine containing compounds has been shown to play a role in their poor pharmacokinetic properties [83]. Because it is likely that this same issue will arise with an amidine or guanidine containing uPA inhibitor, the less basic amiloride scaffold makes an attractive starting point for the design of an orally available uPA inhibitor.

Table 1. Inhibition Constants for uPA Inhibitors Versus Several Serine Proteases. Inhibition Constants are in μM, and are K_is Unless Noted

Compound #	uPA	tPA	Plasmin	Thrombin	Factor Xa	P-Kallikrein	Trypsin	Reference and Comments
1	6.1	>1000	>500	>1000		>2000	120	(77); 4-chloro phenylguanidine
2	6.5	>1000	>500	>1000		>2000	63	(77); 4-trifluoromethyl phenylguanidine
3	2.4	>1,000	>1,000	600	>1,000	-	-	(78,79); WX-293T
4	7.0	>1000					32	(48,49,73); amiloride
5	0.061	>60	17.4	-	-	-	-	(81-84)
6	0.17	52	>100					(81-84)
7	6.1		100					(85)
8	2.2		7.9					(85)
9	5.9	>100	51			23	7.8	(73,85); naphthamidine
11	0.41	4.9	0.39	0.49	1.7	7.2	0.037	(87); WX-UK1
12	0.64	8.7					0.6	(78)
13a	0.036		11	13	3.0		0.15	(88)
13b	0.0077		0.54	0.11	2.1		0.0033	(88)
14	0.0031	>2500	370					(91)
15	3.7	>1000	>1000					(92)
16	0.070	24	>250					(92,93); B623; IC_{50}
17	0.32 0.21	110 16.8	350	20	30		0.44	(92); B428; IC_{50} (74); APC-6860; K_i
18	0.60							(72,76)
19	0.040	48	1.6	4.5		1.4	0.33	(72,76)
20	0.035	25	3.8	3.2		10	1.7	(72,76)
21	0.63	32	2.0	5.6		2.5	0.32	(76)
22	0.040	54	16	>100		7.2	2.1	(76)
23	0.0006	0.68	0.15	0.94		0.04	0.02	(76)
24	0.0009	1.1	0.17	2.2		0.045	0.035	(76)
25	71							(96)
26	2.5 0.31							(96); pH 7.5 (96); pH 6.5
27a	0.008	0.035	0.10	0.32	0.078		0.13	(75,95,101); APC-8696
27b	0.009	8.8	0.11	60	19		0.23	(75,95,101); APC-10302
28a	1.1			6.6	4.6		2.5	(101); APC-6669
28b	2.3			4.0	2.0		3.6	(101); APC-1144
29	31			45			16	(101); APC-10273
30	3.9							(102)
31	0.06							(102)

Despite this advantage, there have been no reports of an amiloride or acyl-guanine containing uPA inhibitor beyond the parent compound.

Two heterocycle-based aryl guanidines have been reported recently by Pfizer [84-87]. The guanidino isoquinoline (5), is a potent inhibitor of uPA, K_i = 61 nM. Although no X-ray



crystallographic studies have been reported for this compound, modeling of the structure suggests **5** should bind in a similar fashion as the 4-chloro phenylguanidine or amiloride. Presumably the 4-chloro group of the isoquinoline should be in close proximity to the Ser195, similar to the 3-amino group in amiloride that forms a hydrogen bond with the Ser195 hydroxyl. The 2-methoxyphenyl substituent on the 7 position of the isoquinoline ring may be directed to the S1β site in a similar fashion as the 6-chloro group of amiloride. Presumably, it occupies more of the site, thereby improving the potency of the compound. The guanidine pyridine structure (**6**) has a K_i is 170 nM. The molecule exhibits 300-fold selectivity for uPA over tPA and 6000-fold selectivity for uPA over plasmin. Again, modeling suggests that the 4-chloro group is near Ser195 and the phenylcarboxylate occupies the S1β site.

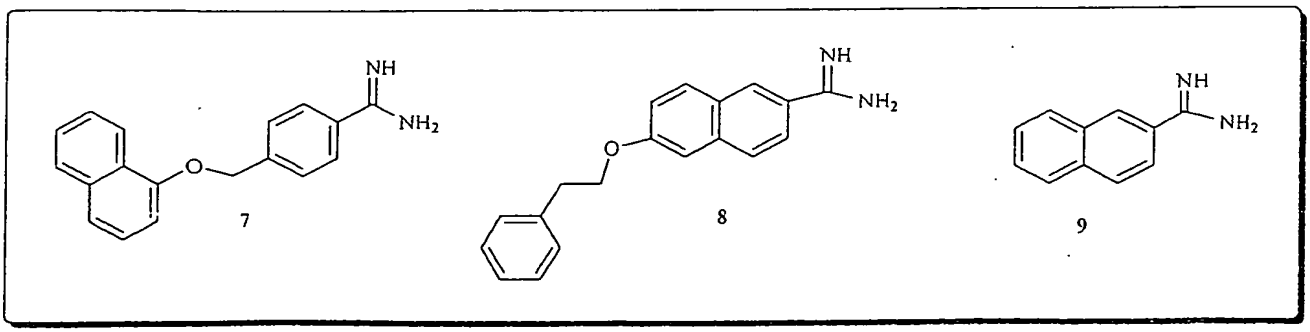
ARYL AMIDINES

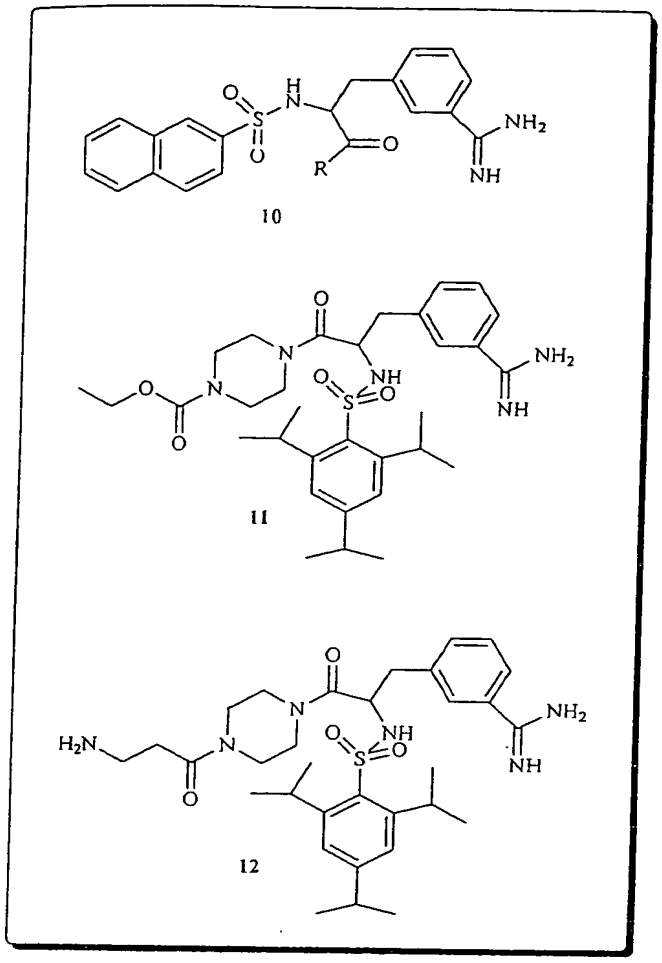
Early Benzamidine and Naphthamidine Compounds

The discovery of substituted benzamidine (**7**, K_i = 6.1 μM) and naphthamidine (**8**, K_i = 2.2 μM) as templates for uPA inhibition was reported by Sturzebecher and Markwardt in 1978 [88]. The uPA inhibitory potency of unsubstituted benzamidine was determined to be between 30 and 180 μM [76,77,82,89]. Both 3- or 4-substituents were tolerated on the benzamidine. Unsubstituted naphthamidine (**9**) exhibits uPA inhibition of 5 μM. Many uPA inhibitors have been based on these aryl-amidines.

Benzamidine

Refined crystal structures of meta-substituted benzamidine-based uPA inhibitors have been described recently by Stuzebecher *et al.* [90]. This particular class of inhibitors (**10**) has been previously shown to inhibit thrombin and trypsin with K_i values in the nanomolar range. Inhibition of uPA, however, was weaker. Replacement of the β-Naphthylsulfonyl group in **10** with the triisopropylphenylsulfonyl group improved the uPA inhibitor potency from 6 to 10-fold. Investigation of the nature of the R group in **10** necessary for potent uPA inhibition led to a series of N-substituted piperazides. The optically active-L-enantiomer of the N-ethoxycarbonyl piperazide (**11**, WX-UK1) exhibited potent uPA inhibition with a K_i = 0.41 μM. This compound, however, exhibited little selectivity for uPA over the other trypsin family proteases: tPA, plasmin, thrombin, factor Xa, plasma kallekrein, and trypsin (Table **1**). A related analog (**12**) also exhibited potent uPA inhibition (K_i = 0.64 μM), but improved selectivity against tPA. However, **12** exhibits no

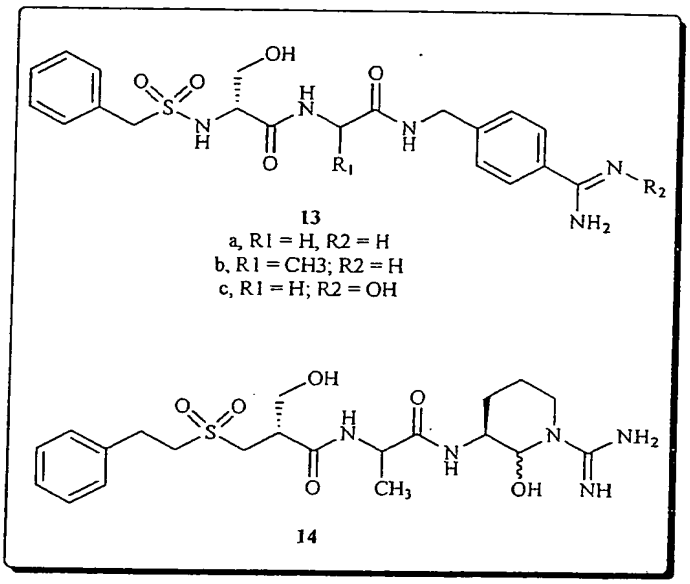




selectivity for uPA versus trypsin (Table 1). The X-ray structure of 12 was analyzed as a complex with uPA [82]. The amidino phenyl moiety binds in the S1 pocket forming the characteristic hydrogen bonds with Asp189. The β-alanine moiety is attached to the piperazine nitrogen and projects toward the indentation between His99 and the catalytic triad residue His57, forming a hydrogen bond with the Tyr94 hydroxyl group. One of the isopropyl groups of the triisopropylphenylsulfonyl moiety projects toward a small dimple situated to the left of His99 and at the edge of the S4 site (Fig. 1).

This same group has recently reported on a series of 4-substituted benzamidine (4-amidinobenzylamine) structures [91] based loosely on the structure of the thrombin inhibitor melagatran [92,93]. For these peptidomimetic compounds, the research team started with 4-amidinobenzylamine-Gly-Glu. Substitution of the P3 Glu residue with d-Ser and capping the d-Ser amino group with a benzylsulfonyl group improved potency 60-fold, to K_i = 36 nM versus uPA (13a). Substitution of the P2 with Ala (13b) or Pro further increased potency (7.7 and 13 nM K_i, respectively), but at the cost of selectivity (Table 1). These peptidomimetics had poor pharmacokinetic properties and were rapidly eliminated from plasma when administered parenterally. Some improvement in the half-life of the compounds was achieved by creating the hydroxyamidine analogue (13c) of compound 13a. This prodrug is rapidly converted to the active compound *in vivo*.

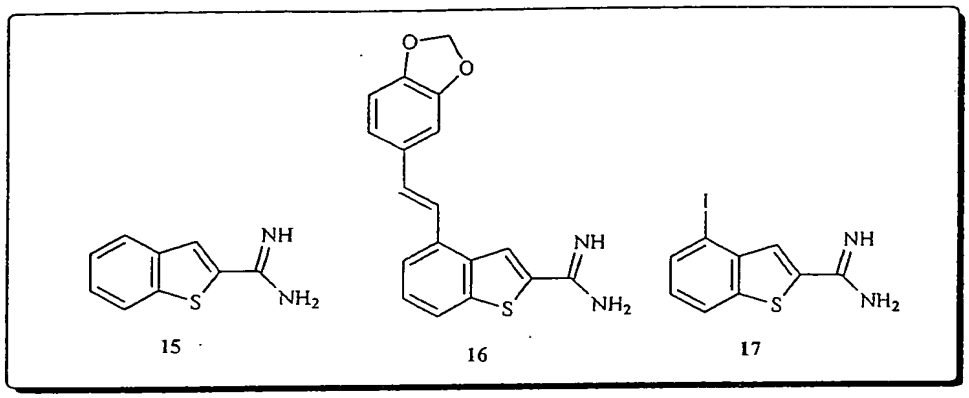
The discovery of these compounds in many ways is similar to another, preceeding, peptidomimetic strategy that had been followed by investigators at Corvas [94]. This group also found P2 and P3 were optimally Ala and d-Ser. However, the P1-substituents in this series were argininals, not amidinobenzylamines (14). Similarly, a prodrug strategy was also used, resulting in compounds with longer *in vivo* half-lives.



Benzo[b]thiophene-2-Carboxamidine

A major improvement in the potency of uPA aryl-amidine inhibitors was described in 1993 by Littlefield and coworkers [95,96]. They used benzo[b]thiophene-2-carboxamidine (15) as the basis for their inhibitors. The unsubstituted benzo[b]thiophene-2-carboxamidine has the nearly the same uPA potency, K_i = 3.7 μM, as naphthamidine, K_i = 5 μM. Substitution at the 4 position dramatically improved the compounds, where the best compound exhibited uPA inhibition of 70 nM (16). The 4-iodo compound, 17, is also potent with a K_i = 0.32 μM. Substitution at the 5-position [97] of the benzothiophene template provided compounds that were approximately 10-fold less potent than the 4-substituted derivatives. This 4-substituted series of inhibitors also exhibited greater than 300 to 1000-fold selectivity for uPA over tPA or plasmin. Compounds 16 and 17 were also tested for their ability to inhibit uPA catalyzed cellular basement membrane degradation. The cell surface uPA on HT-1080 human fibrosarcoma cells was inhibited with IC_{50} = 1.5 μM and IC_{50} = 0.39 μM, respectively [96].

The structure of 17 complexed with uPA, shown in (Fig. 3), reveals a strong interaction of the amidine moiety with the Asp189 carboxylate and hydrogen bond interactions with the Ser190 side chain oxygen and the Gly218 carbonyl oxygen [75,77]. There are additional hydrophobic contacts of the aromatic ring with the protein residues that define the S1 pocket. These interactions alone confer improved uPA



inhibition versus benzamidine, approximately 3.7 μM for the unsubstituted 2-amidino-Benzo[b]thiophene (**15**). However, **17** exhibited a 10-fold increase in potency over the parent compound indicating that the 4-iodo substituent must be involved in additional interactions with the protein. Indeed, the 4-iodo group is directed toward the S1β pocket in a manner similar to the chloro-substituent of amiloride. Compound **16** is approximately 10-fold more potent in uPA inhibition (K_i = 0.07 μM) than **17**, suggesting that this larger group may better occupy the S1β site than does the 4-iodo compound.

Modeling of the **17**-uPA complex (Fig. 3) suggests other substitutions on the aromatic ring could result in additional potency improvements. Positions 5 and 6 are also potential sites for modification since these positions on the aromatic ring are not buried in the S1 pocket. However, uPA inhibition data published in an Eisai patent [97] of approximately 100 compounds with substitutions at position 5 or positions 4 and 5 in the benzo(b)thiophene series shows no potency improvements over compounds **16** and **17**. Recently benzo(b) thiophene analogs substituted in position 6 as well as disubstituted benzo[b]thiophene analogs substituted in positions 4 and 6 have been reported [98]. These analogs exhibited uPA inhibition between 100 nM and 3μM.

6,8 Disubstituted Naphthamidines

2-Naphthamidine served as the basis for a structure-based drug design program at Abbott Laboratories [75,79]. The structure of unsubstituted naphthamidine suggested that substitutions at positions 6, 7, or 8 would be the most likely to yield improvements in the naphthamidine based series (Fig. 3). Position 6-substituents would be directed towards the catalytic Ser195 as well as the variable loop (containing Asp60A). Position 8 is directed towards the S1β pocket that had been previously used with good effect in the benzo(b)thiophene series. The remaining positions, 1,3,4,5 are all buried in the S1 pocket and could likely accommodate no (or perhaps very small) substituents. The peptide binding region, S2 and S4 sites, or the substrate-binding groove do not appear to be easily accessible from any position on the naphthamidine template (Figs. 1, 3).

The fundamental premise of the naphthamidine design strategy was to first find substituents at each position that by themselves provide potent and selective inhibition of uPA, and then combine these substituents in a single naphthamidine based molecule that would exhibit improved uPA inhibition and selectivity over each monosubstituted naphthamidine from which it was derived. This strategy was feasible, and ultimately proved successful because the naphthamidine core moved little when substituted at either the 6 or 8 position.

Position 8-substituents are directed to the S1β pocket. This site contains a water molecule that forms a hydrogen-bonded network to several residues within the S1β pocket including the Ser146 side chain hydroxyl, Lys143 side chain amine and the Gln192 side chain amide carbonyl. As demonstrated in the benzo[b]thiophene-2-carboxamidine series, large polarizable groups like iodine or larger groups like the methylenedioxo phenyl group can fill S1β the pocket displacing the water and enhancing potency 10 to 30-fold. Alternatively, position 8-substituent groups could be used that form hydrogen bonds with the water in the S1β pocket and also improve potency.

The initial inhibitors included a naphthamidine template substituted in either position 8 alone (8-mono substituted series) or substituted in positions 7 and 8 (7,8 disubstituted series). Evaluation of approximately 50 – 100 7,8 disubstituted naphthamidines led to the discovery of 7-methoxy-8-acetamidoxy-2-napthamidine (**18**). This compound exhibited comparable uPA inhibitor potency (K_i= 0.60 μM) to **17**. Analysis of the X-ray structure of this compound revealed a water mediated hydrogen bond from the amide carbonyl of the inhibitor side chain in position 8 and the Lys-143 residue in the S1β pocket. The effect of substitution at position 7 was variable, but typically decreased potency slightly. Crystal structures revealed that the 7-substituents were directed towards solvent, explaining their lack of effect on compound potency.

Using the structural data obtained from the 7-methoxy-8-acetamidoxy-2-naphthamidine (**18**), a series of alkyl carbamates substituted in position 8 was designed. Evaluation of this series of compounds for uPA inhibition revealed that the 8-methylcarbamate-2-naphthamidine (**19**) was a potent inhibitor of uPA, K_i = 0.04 μM, approximately 10-fold more potent than the 7-methoxy-8-acetamidoxy-2-naphthamidine and 100-fold more potent than 2-naphthamidine. Analysis of the inhibitor-protein complex shows the position 8-substituent residing in the S1β pocket and the carbonyl of the carbamate moiety involved in a water mediated hydrogen bond with the Lys143 side chain amine and the Gln192 side chain amide carbonyl group. Moreover, the NH of the

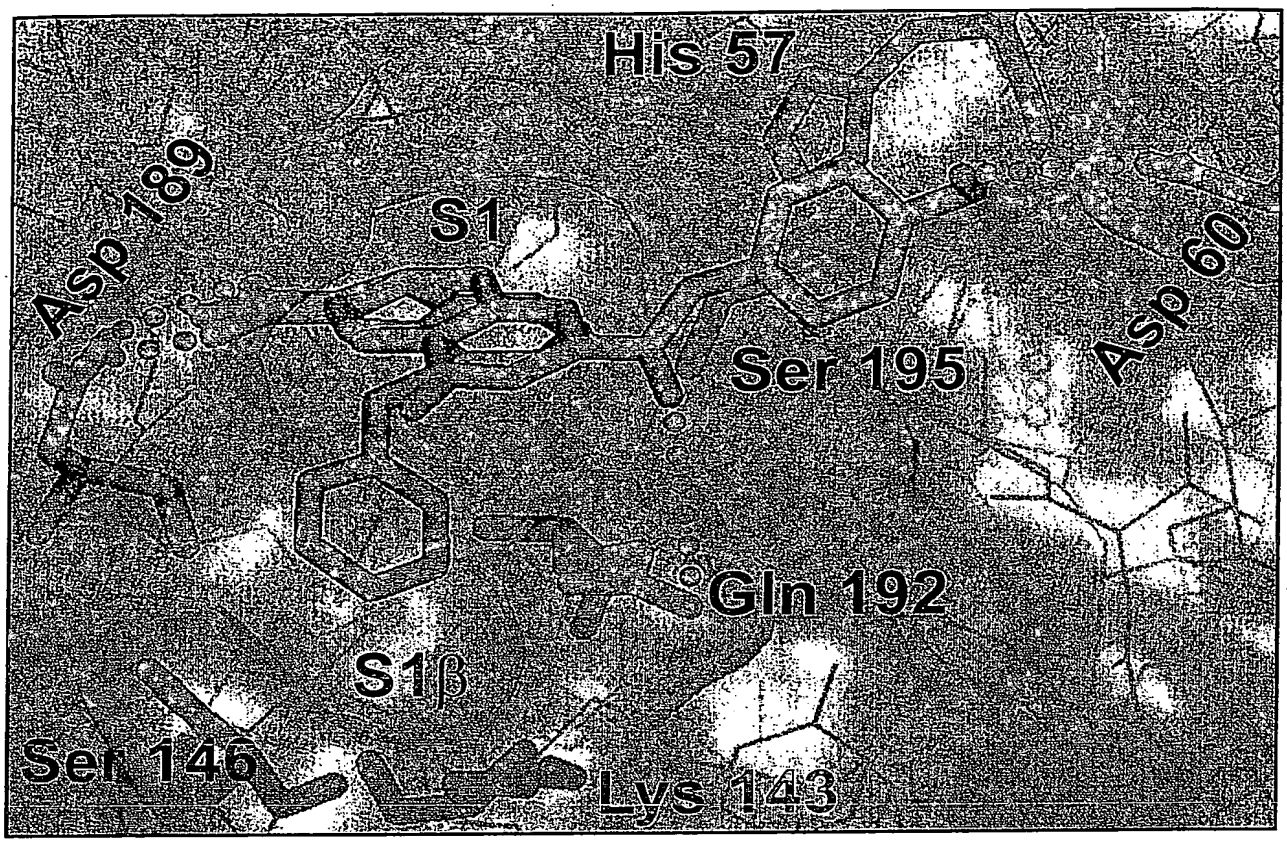


Fig. (3). An overlay of 17 (B428, orange) and 23 (magenta) in the active site of uPA. Both form hydrogen bonds between their amidine groups and Asp189. Both also have substitutions that fill the S1β pocket, 4-Iodo for 17 and 8-aminopyridine for 23. The 6-substitution of 23 also makes hydrogen bonds with Gln192 and Asp60A in the variable loop. The naphthamidine, depicted here with compound 23, remains in the same position whether unsubstituted, 6-mono, 8-mono, or 6,8 disubstituted.

carbamate group is involved in a hydrogen bond to the Gly216 residue. We investigated the size of the group tolerated in the S1β pocket by further modifying the position 8-substituents. The 8-aminopyrimidine analog (**20**) exhibited the same uPA inhibitor potency, K_i=0.03 μM, as the 8-methylcarbamte analog (compound 19). The crystal structure of **19** [75] reveals the position 8 amino pyrimidine group fully occupying the S1β pocket while maintaining the inhibitor amine NH hydrogen bond to the Gly216 residue of the protein. Compounds **19** and **20** also exhibited good selectivity for uPA over other trypsin family serine proteases including plasmin, tPA, plasma kallikrein, thrombin and trypsin (50-fold, Table 1).

Position 6-substituents may be directed to several remote sites within the active site. One potential site is the unique Asp60A residue (shown in Fig. 1) where a basic substituent on the inhibitor may form a hydrogen-bond interaction with the side carboxylate of the Asp. Alternatively, a position 6-substituent could be directed toward the cationic loop, containing two arginine residues, Arg35 and Arg37, whereby a salt bridge or hydrogen bond interaction with an appropriately functionalized substituent, for example a carboxylate or sulfonate, would improve the potency.

The initial inhibitor design utilized an amide linker between the naphthamidine and the position 6-substituent to direct substituents to remote regions of the active site. We initially evaluated the 6-phenylamide-linked 2-naphthamidine (**21**) and found it to have uPA inhibitor potency (K_i = 0.63 μM) nearly equal to **17**,(K_i = 0.31 μM). Analysis of the X-ray structure of the 6-phenyl amide analog revealed a hydrogen bond interaction between the carbonyl of the amide link in the inhibitor and the amide of the Gln192 side chain. The amidine moiety is characteristically bound in the S1 pocket as has been discussed previously. The phenyl group of the inhibitor maintains a favorable van der Waals contact with the protein. Moreover, the para-position of the phenyl ring appears to be the most favorable site for attaching substituents that might interact with the Asp60A residue. This hypothesis was tested by evaluating a series of para-substituted amine functionalities on the phenyl ring for uPA inhibitor potency. The optimal substitution was the para aminomethyl substituent (**22**), on the phenyl ring producing a uPA inhibitor with a K_i = 0.03 μM. An X-ray structure of **22** shows the carbonyl of the amide link maintaining the hydrogen bond interaction with Gln192 side chain carboxamide, the phenyl ring maintaining favorable van der Waals contact with the protein and the para-aminomethyl group forming a favorable hydrogen bond interaction with the Asp60A. The selectivity for **22** was excellent for uPA over other trypsin family serine proteases (Table 1). Thus, directing a substituent to a particular remote site, in this case

the Asp60A residue, within the active site can indeed improve uPA inhibitor potency and selectivity.

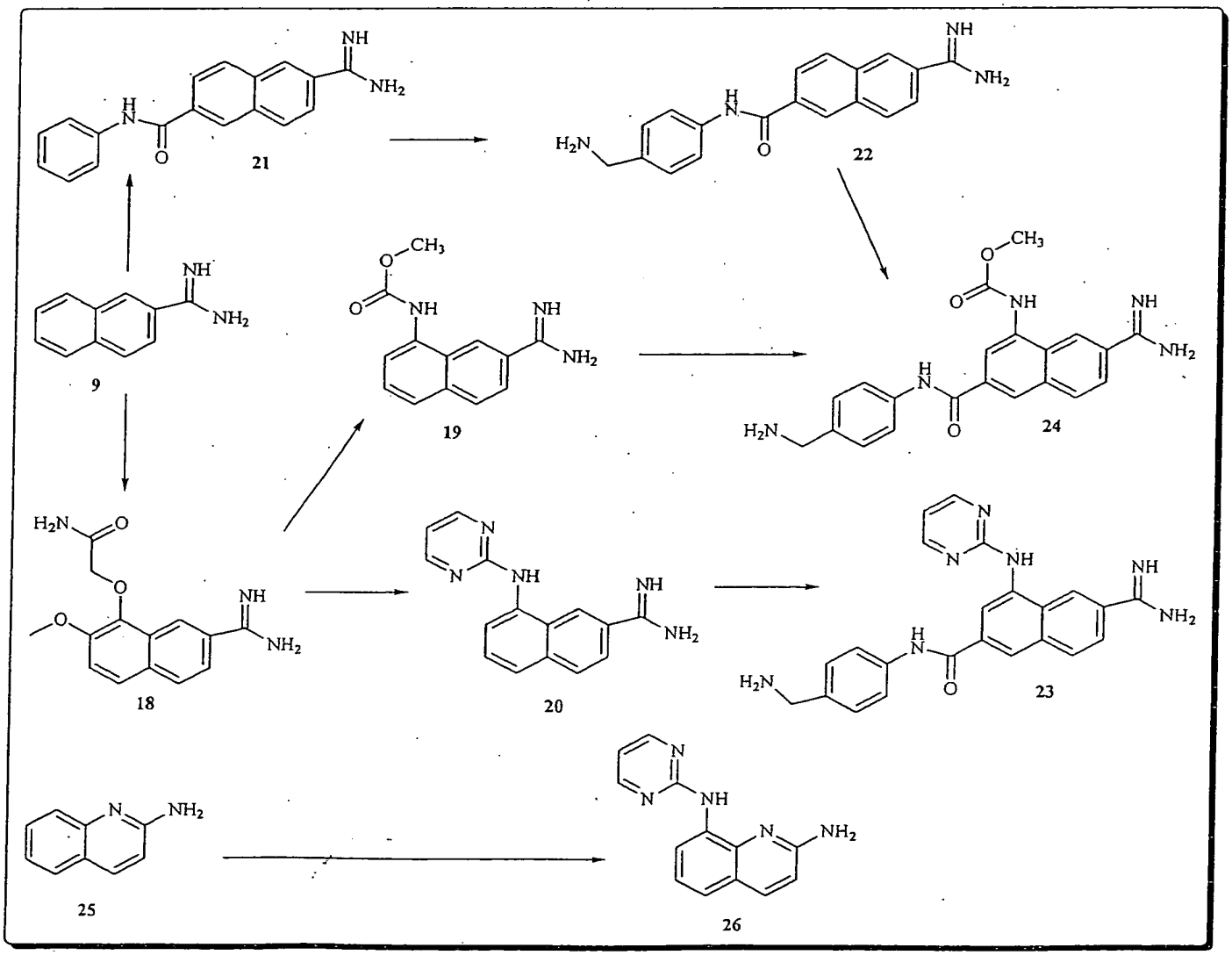
The 6,8 disubstituted naphthamidine inhibitors, 23 and 24 were more potent than any of the mono-substituted compounds with a K_i values versus uPA of 0.0006 and 0.0009 μM respectively. These are the most potent uPA inhibitors reported to date. Compounds 23 and 24 exhibited excellent selectivity for uPA over related trypsin family serine proteases including plasmin, tPA, plasma kallikrein, trypsin, and thrombin. Compound 23 was evaluated for its ability to inhibit cell surface uPA-mediated basement membrane degradation in a human pancreatic cancer cell line that overexpresses uPA (MiaPaCa) and showed potent inhibition (IC_{50} = 3 nM) approximately 500-fold greater than 17.

The crystal structure of 23 is shown in (Fig. 3) as an overlay with 17. The most potent disubstituted naphthamidines maintain the four important interactions of the inhibitor with the protein; (1) The favorable hydrogen bonding interactions of the para-aminomethyl substituent with the Asp60A residue; (2) The amide linker carbonyl hydrogen bond with the Gln192 side chain amide group, orienting the phenyl ring toward the Asp60A residue in the protein as well as anchoring the inhibitor to the protein; (3) maximum occupancy of the 8-position 2-aminopyrimidine moiety in the S1β pocket and (4) the strong amidine-Asp189 salt bridge interaction in the S1 site.

Naphthamidine Replacements: 2-Amino-Quinolines and 2-Aminobenzimidazoles

Scientists at Abbott used novel crystallographic [99] or NMR [100] based screening methods to look for previously undiscovered S1 site binding moieties. Using these techniques they were able to identify a number of pharmocophores that bound in the S1 pocket, and had lower pKas (8.5 vs. 12) than the aryl amidines. The lower pKa should increase the possibility that the resulting compounds would be orally bioavailable. These pharmacophores are exemplified by the 2-aminoquinolines [99] (25) and 2-aminobenzimidazoles [101].

While the initial lead, 25, displayed only modest potency vs. uPA (K_i = 71 μM) Neinaber *et al.*, were able to use the S1β pocket as they had with the naphthamidine series to significantly increase potency (26, Fig. 2). The resulting N-



pyrimidyl 2-amino quinoline has a K_i of 2.5 μM vs. uPA at pH = 7.4 (0.31 nM at pH = 6.5). This compound, **26**, also displayed improved oral bioavailability (F = 38 %, rat) versus the naphthamidine analogue (**20**, F = 0%, rat).

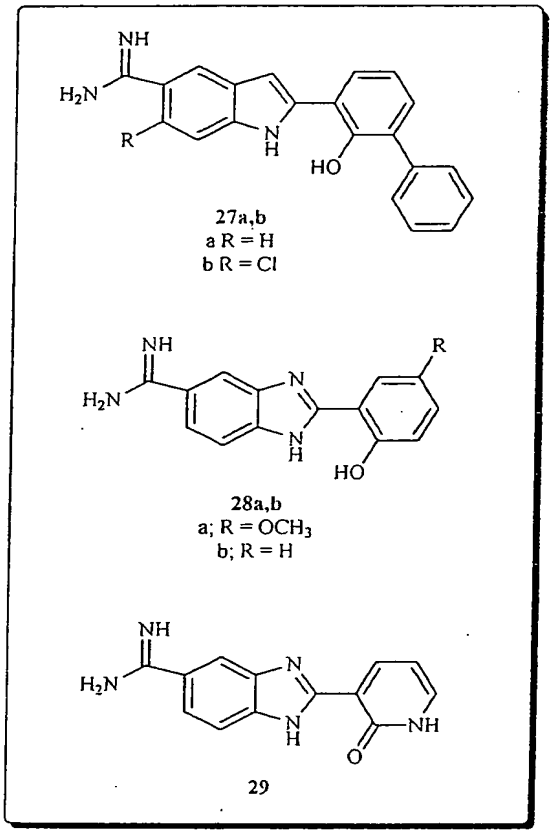
Amidino Indoles and Amidino Benzimidazoles

Recently, Axys Pharmaceuticals (now Celera) reported the development of small molecule uPA inhibitors utilizing a structure-based approach based on an amidinobenzimidazole or amidinoindole templates [78,102-104]. Compound 27a, an amidinobenzimidazole based inhibitor, exhibited uPA inhibition of 8 nM, but only modest (less than 20-fold) selectivity for tPA, factor X_a, plasmin, and trypsin (Table 1). Axys scientists proposed more selective uPA inhibitors within this class of compounds could be realized by the introduction of substituents on the aromatic moiety of the amidine [77,78]. Compound 27b, an amidinoindole based inhibitor, exhibited uPA inhibition of 9 nM and improved selectivity for uPA over tPA (1000 X), factor Xa (2000 X), thrombin (6000 X), and porcine kallekrein (90 X). However, less than 10-fold selectivity for uPA over plasmin, trypsin and Factor VII_a was exhibited by 27b. The observed improvement in selectivity for 27b over 27a is due, in part, to the chloro substituent adjacent to the amidine on the indole nucleus. In both Ala190 (factor Xa, thrombin, plasma-kallikrein) and Ser190 (uPA, Plasmin, trypsin, factor VIIa) containing enzymes, there is a water-mediated hydrogen bond between the N^1H of the amidine of the inhibitor and the protein residue in the S1 subsite. The substitution on the position adjacent to the amidine moiety with chlorine causes the displacement of the water in either Ala190 or Ser190 enzymes. The observed selectivity between the enzymes is due the hydrogen bond between N^1H^1 of the amidine and the Ser190 side chain, while a hydrogen bond in Ala190 enzymes is not possible. This proposal was confirmed by X-ray crystallography, which provided evidence for the displacement of the water by the chloro. The Axys work provided an alternative path for improving inhibitor selectivity and illustrated that selectivity improvements can be achieved through modifications of the S1 subsite without drastically increasing overall molecular weight.

Using a large number of high-resolution structures, Katz and coworkers [104] were able to distinguish a set of compounds that form an unusual network of short hydrogen bonds between a water (bound in the oxy-anion hole), their compounds and the catalytic serine (**28a, 28b**). What makes these compounds very interesting is that the interactions of these short hydrogen bonds are strong enough to pull the amidine moiety away from Asp189, and disrupt the canonical hydrogen bonds that occur at the pocket base (Fig. **4**). There are no direct hydrogen bonds between Asp189 and the

Fig. (**4**). An overlay of 28a (APC-6669, pink) with 29 (APC-10273, green). Although both are structurally similar, 28a makes a network of short hydrogen bonds with Ser195 and a water in the oxyanion hole (not shown), while 29 does not. The result of the short Ser195 hydrogen bond network in 28a is to pull its amidine away from Asp189 to a distance of 4 Angstroms. In spite of this poor amidine interaction, 28a is more potent than is 29.

amidine of compounds 28a,b as there are in 29, where the inhibitor does not make the network of tight hydrogen bonds at the catalytic Ser195. In spite of the loss of the Asp189 hydrogen bond, 28a,b are more potent than more typical (29) counterparts. This leaves open two possibilities: (1) compounds with no amidine moiety can be designed that would be as potent as their more polar counterparts, thus improving pharmacokinetic parameters; or (2) reintroduction of the Asp189 hydrogen bond, while maintaining the short hydrogen bond network at Ser195, could result in super-potent compounds.



Thiophene 2-Carboxamidines

Recently, researchers at 3-Dimensional Pharmaceuticals [105,106] have discovered a new template on which to base a uPA inhibitor program. Screening of amidine libraries revealed that 5-methylthiothiophene-2 carboxamidine inhibited uPA with K_i = 6 μM, nearly equipotent with 2-naphthamidine[106]. Modeling, based on 17 suggested that the 5-position of the thiophene would be nearly equivalent to the 4-postion of benzamidine. However, this modeling also suggested that substitutions at the 5-position would change the orientation of the thiophene ring within the S1 pocket. Therefore this site would need to be optimized first, as all other substitutions would depend on the 4-substituent. The SAR supports this, and the contribution of substituents at the 4-position varies considerably with the 5-position substituent.

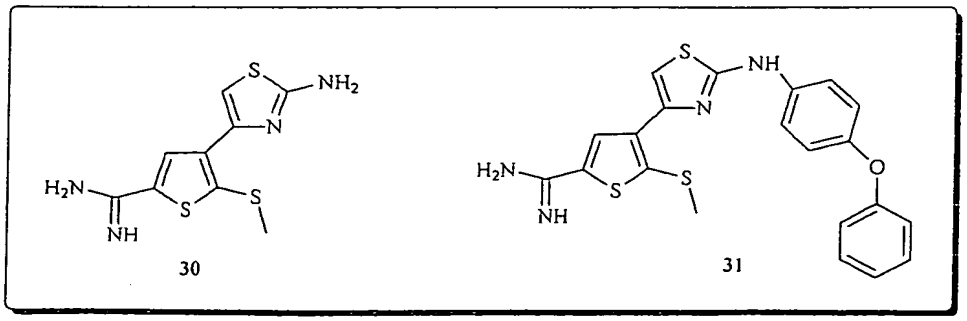
Elaboration of the 4-position suggested that small aliphatic groups, methyl or ethyl, were as potent as the original S-methyl lead. It is interesting that hydrogen at this position leads to a considerable, 50-fold, decrease in potency. Larger, aromatic groups are likewise not tolerated. Although the methyl and ethyl groups do not improve potency over the S-methyl, they have the advantage of a nearly 10-fold increase in aqueous solubility.

Modeling of the thiophene 2-carboxamidine suggested that four position substituents might provide access to the S1β pocket that had been used successfully in the 4-substituted benzo[b]thiophene-2-carboxamidine or the 8-substituted 2-naphthamidine series[105]. Chemical elaboration of this hypothesis proceeded based on an aminothiazole substituent at the 4-position (30). Further elaboration via substitutions on the N–position of the aminothiazole proved valuable, with many compounds discovered with K_i values less than 1 μM. Aromatic groups proved particularly potent, and a biaryl ether substituent had a K_i = 60 nM (31). No enzyme selectivity data and no structural data have been reported for these compounds.

CONCLUSION

The development of potent and selective small molecule inhibitors of uPA possessing either an aryl amidine template or an aryl guanidine template has been realized. Utilizing available structural information, medicinal chemists have adopted different approaches, most notably in the aryl amidine template, to achieve improved potency and selectivity. This has been a long and difficult process. Studies of large numbers of aryl guanidine, aryl amidine and aryl acylguanidine templates have led to relatively few small molecule uPA inhibitors exhibiting submicromolar uPA inhibition.

The advent of routine X-ray analysis of enzyme-inhibitor complexes has allowed a detailed study of important



inhibitor-protein interactions of existing uPA inhibitors. The newly available structural data from different classes of inhibitors have enabled investigators to discern the most common and favorable protein-inhibitor interactions. This knowledge has, in turn allowed the development of more potent and selective uPA inhibitors.

While the goals of potency and selectivity have been achieved, the pharmacokinetic properties of many of these compounds are not sufficient to allow them to enter clinical (or even pre-clinical *in vivo*) trials. The basic amidine functionality is typically a liability for both oral bioavailability and elimination half-life. Approaches borrowed from other serine protease inhibitor programs, either pro-drugs, or less basic amidine replacements are starting to prove successful. The achievement of uPA inhibitors with desirable pharmacokinetic properties will enable investigators to achieve their ultimate goal of defining the clinical utility of uPA inhibition.

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ABBREVIATIONS

uPA	=	Urokinase-Type plasminogen activator or urokinase
tPA	=	Tissue plasminogen activator
uPAR, CD87	=	Urokinase receptor
QSAR	=	Quantitative Structure-Activity Relationship

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